

Glutamate transporter type 3 attenuates the activation of N-methyl-D-aspartate receptors co-expressed in *Xenopus* oocytes

Zhiyi Zuo* and Hongyu Fang

Department of Anesthesiology, University of Virginia Health System, Charlottesville, Virginia 22908-0710, USA

*Author for correspondence (e-mail: zz3c@virginia.edu)

Accepted 14 March 2005

Summary

We studied the regulation of N-methyl-D-aspartate receptor (NMDAR) current/activation by glutamate transporter type 3 (EAAT3), a neuronal EAAT *in vivo*, in the restricted extracellular space of a biological model. This model involved co-expressing EAAT3 and NMDAR (composed of NMDAR1-1a and NMDAR2A) in *Xenopus* oocytes. The NMDAR current was reduced in the co-expression oocytes but not in oocytes expressing NMDAR only when the flow of glutamate-containing superfusate was stopped. The degree of this current reduction was glutamate concentration-dependent. No reduction of NMDAR current was observed in Na⁺-free solution or when NMDA, a non-substrate for EAATs, was used as the agonist for NMDAR. In the continuous flow experiments, the dose–response curve of glutamate-induced current was

shifted to the right-hand side in co-expression oocytes compared with oocytes expressing NMDAR alone. The degree of this shift depended on the abundance of EAAT3 in the co-expression oocytes. Thus, the glutamate concentrations sensed by NMDAR locally were lower than those in the superfusates. These results suggest that EAAT3 regulates the amplitude of NMDAR currents at pre-saturated concentrations of glutamate to EAAT3. Thus, EAATs, by rapidly regulating glutamate concentrations near NMDAR, modulate NMDAR current/activation.

Key words: glutamate receptor, glutamate transporter, neurotransmission, oocytes.

Introduction

Glutamate is a major excitatory neurotransmitter. Since no enzymes have been found to metabolize glutamate extracellularly, glutamate can be cleared from the synaptic cleft in two ways: diffusing away and being uptaken into cells by glutamate transporters (also called excitatory amino acid transporters, EAATs) (Clements et al., 1992; Danbolt, 2001). Five EAATs have been characterized so far: EAAT 1–5 (Danbolt, 2001). In rat, EAAT1 and 2 are expressed in glial cells and EAAT3 and 4 are expressed mainly in the post-synaptic elements, such as dendritic shafts, spines and axons of neurons (Rothstein et al., 1994). EAAT5 is mainly distributed in glial cells or neurons of the retina (Arriza et al., 1997).

The regulation of extracellular glutamate homeostasis by EAATs has been investigated by two approaches: molecular biology manipulations, in which the expression of a selective EAAT is disrupted, and pharmacological blockade, in which transporter function is inhibited with appropriate inhibitors. By using molecular biology technique, it has been shown that mice that lack EAAT1 or EAAT2 expression had increased extracellular concentrations of glutamate and that the animals were susceptible to seizures and excitotoxic cell death (Rothstein et al., 1996). Although such an approach provides important functional information on EAATs to regulate

extracellular concentrations of glutamate in the central nervous system, the chronic inhibition of the gene expression may induce compensatory mechanisms and will not provide information on the dynamics of extracellular glutamate homeostasis in response to acute disruption of the uptake system.

Pharmacological blockade of EAATs has been used to study the role of EAATs in the dynamics of glutamate homeostasis. The inhibition of EAAT activity has been shown to prolong the glutamate-induced current, leading to a slowed excitatory post-synaptic current (EPSC) decay at some synapses (Barbour et al., 1994; Diamond and Jahr, 1997; Kinney et al., 1997; Mennerick and Zorumski, 1994; Otis et al., 1996; Takahashi et al., 1995; Tong and Jahr, 1994). A recent study further suggests that glutamate translocation by EAATs is important in control of EPSC decay (Mennerick et al., 1999).

We used a different approach, co-expression of N-methyl-D-aspartate receptor (NMDAR) and EAATs, to investigate the role of EAATs in the control of NMDAR activation. We artificially expressed NMDAR with or without a neuronal EAAT, EAAT3, in *Xenopus* oocytes. This approach allowed us to compare the activation of NMDAR in the presence or absence of EAAT3 without the need to use multiple inhibitors for EAATs or glutamate receptors. By using this model,

we tested the hypothesis that EAATs regulate NMDAR activation/current induced by glutamate.

Materials and methods

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Virginia. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised in 1996. All efforts were made to minimize the number of animals used and their suffering.

Materials

All reagents, unless specified below, were obtained from Sigma Chemical (St Louis, MO, USA).

cRNA preparation

Rat EAAT3 cDNA in a commercial plasmid vector (BluescriptSKM) was provided by Mattias A. Hediger. Rat NMDAR1-1a in pBS SK(–) and rat NMDAR2A in pBS SK(+) were from Steve F. Heinemann. The cDNAs were linearized with restriction enzymes that were suggested by the people who made the constructs. The capped cRNAs were transcribed using a commercial T7 polymerase (Ambion, Austin, TX, USA).

Oocyte preparation and injection

These procedures were performed as we described before (Do et al., 2001, 2002b; Fang et al., 2002). Briefly, one day before cRNA injection, stage V–VI oocytes were isolated from adult female *Xenopus laevis* (Daudin) frogs (Ann Arbor, MI, USA) anesthetized with 0.2% 3-aminobenzoic acid ethyl ester. After being surgically removed from the frog, oocytes were defolliculated with 20 mg collagenase (Type 1a) in 20 ml of Ca²⁺ free OR2 solution (containing in mmol l^{–1}: NaCl 82.5, KCl 2.0, MgCl₂ 1.0, and HEPES 5.0, pH adjusted to 7.4) for 2 h at room temperature (22°C). Oocytes were injected (Drummond ‘Nanoject’, Drummond Scientific Co., Broomall, PA, USA) with 40 ng cRNA of EAAT3 or 10 or 40 ng cRNA of NMDAR1-1a and NMDAR2A (1:4 weight ratio). Oocytes were then incubated at 16°C in modified Barth’s solution (containing in mmol l^{–1}: NaCl 88, KCl 1, NaHCO₃ 2.4, CaCl₂ 0.41, MgSO₄ 0.82, Ca(NO₃)₂ 0.3, gentamicin 0.1, and HEPES 15, pH adjusted to 7.4) before voltage clamping was performed.

Electrophysiological recordings

As we described before (Do et al., 2002b; Fang et al., 2002), 4–5 days after injection of the cRNA, oocytes were superfused by gravity flow with Mg²⁺- and Ca²⁺-free Ringer’s solution (containing in mmol l^{–1}: NaCl 96, KCl 2, BaCl₂ 1.8, and HEPES 10, pH adjusted to 7.5) containing glycine 10 μmol l^{–1}. The flow is about 3 ml min^{–1} and the oocyte chamber volume is about 1 ml. Clamping microelectrodes were pulled from capillary glass (10 μl Drummond Microdispenser, Drummond

Scientific Company, Broomall, PA, USA) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA, USA). Electrodes were broken at the tip whose diameter was approximately 10 μm and filled with 3 mol l^{–1} KCl obtaining resistance of 3 MΩ. Oocytes were voltage-clamped using a two-electrode voltage clamp amplifier (OC725A; Warner Corporation, New Haven, CT, USA), which was connected to a DAS-8A/D conversion board (Keithley-Metrabyte, Taunton, MA, USA) on an IBM-compatible PC. Data acquisition and analysis were performed using the OoClamp program (Durieux, 1993). Current was examined for 70 s (25 s of application of glutamate or NMDA, 45 s of recovery with a glutamate- and NMDA-free superfusate) at a holding potential of –70 mV. Flow-stopped experiments, as modified from a previous study (Supplisson and Bergman, 1997), were performed by applying glutamate or NMDA for 25 s and then flow stopping for 15 s before the flow was resumed for 5 s with a glutamate- or NMDA-containing solution followed by a 35 s of recovery with a glutamate- and NMDA-free superfusate. At least 2 min interval time was allowed after each measurement. Response was quantified by measuring the peak current using OoClamp program. All experiments were performed at room temperature.

Administration of experimental chemicals

Since EAATs are Na⁺-co-transporters, in some experiments Na⁺ in the bath solution was replaced by Li⁺ to inhibit the EAAT3 function. To prevent NMDAR activation in the co-expression oocytes, glycine-free Ringer’s solution containing 5 mmol l^{–1} MgCl₂ was used.

Statistical analysis

Due to the variation in the expression level of EAAT3 and NMDAR proteins in oocytes, glutamate- or NMDA-induced response was normalized to the maximal response of the oocytes to the agents (300 μmol l^{–1} glutamate or 1 mmol l^{–1} NMDA). Results are mean ±s.d. from 6–16 oocytes from at least three frogs. Statistical analysis was performed by unpaired *t*-test. A *P*<0.05 was accepted as significant. EC₅₀ was derived by analyzing data with Graphpad Prism 3.0 (Graphpad Software, Inc, San Diego, CA, USA).

Results

Expression of NMDA receptors and EAAT3

L-glutamate and/or glycine did not induce any current in oocytes uninjected or injected with water (solvent for mRNA of EAAT3, NMDAR1-1a and NMDAR2A) (data not shown). However, oocytes injected with mRNA of EAAT3 or the combination of NMDAR1-1a and NMDAR2A showed inward currents after application of L-glutamate. The responses were concentration-dependent (Fig. 1). The EC₅₀ of EAAT3 and NMDAR for L-glutamate was determined to be 31.8±6.5 μmol l^{–1} (*N*=12) and 3.58±0.87 μmol l^{–1} (*N*=16), respectively, similar to those reported in the literature (Do et al., 2002a,b; Supplisson and Bergman, 1997). As a control experiment, L-glutamate (300 μmol l^{–1}) did not induce any

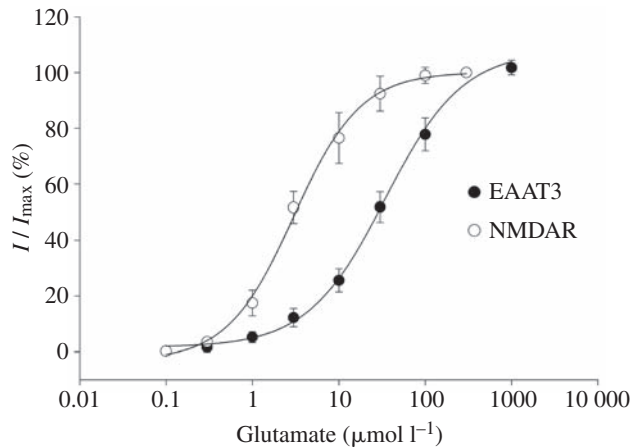


Fig. 1. Glutamate dose–response curves of NMDAR (open circle) and EAAT3 (filled circle). Oocytes were injected with 40 ng of NMDAR mRNA or 40 ng of EAAT3 mRNA. Results are means \pm S.D. ($N=16$ for NMDAR and $N=12$ for EAAT3) of the percentage of the currents induced by various concentrations of glutamate ($\mu\text{mol l}^{-1}$) in the current induced by $300 \mu\text{mol l}^{-1}$ of glutamate (I_{max}).

current in oocytes expressing EAAT3 under Na^+ -free condition (Na^+ was replaced by Li^+) and in oocytes expressing NMDAR in Mg^{2+} -containing and glycine-free solution (data not shown).

Reduction of NMDAR currents in EAAT3⁺ oocytes under stopped-flow condition

In oocytes expressing NMDAR only (EAAT3⁻), the glutamate-induced current remained stable when the flow of the L-glutamate-containing superfusate was stopped. By contrast, co-expressing oocytes developed a marked decrease of the inward current when the superfusion was stopped (Fig. 2). Reestablishing the flow restored the full amplitude of the current (Figs 2 and 3). This phenomenon is called stopped-flow reduction of NMDAR current in this paper.

Since the above results suggest that the stopped-flow reduction of NMDAR current is due to the expression of EAAT3 in the co-expressing oocytes, further experiments to determine whether normal function of EAAT3 is important for

this phenomenon. It is known that EAATs are Na^+ -co-transporters and their transport functions are abolished in a Na^+ -free solution (usually replacing Na^+ with Li^+) (Mennerick and Zorumski, 1994; Roskoski, 1979; Zuo, 2001). Consistent with this idea, L-glutamate in a Na^+ -free and Li^+ -containing solution did not induce any current in oocytes expressing EAAT3 only (data not shown). When the same solution was applied to co-expressing oocytes, no stopped-flow reduction of NMDAR current was observed (Fig. 3). NMDA is an agonist for NMDAR but not a substrate for EAATs (Jabaudon et al., 1999). Consistent with this conclusion, in our study, NMDA induced an inward current in oocytes expressing NMDAR but not in oocytes expressing EAAT3 only (data not shown). When NMDA in Na^+ -containing solution was used to superfuse the co-expressing oocytes, no stopped-flow reduction of NMDAR current was observed (Fig. 3).

The degree of stopped-flow reduction of NMDAR current depended on the concentrations of glutamate in the superfusates (Fig. 4). When glutamate concentrations were more than $100 \mu\text{mol l}^{-1}$, stopping the flow produced a small reduction or no reduction at all (Fig. 4). This phenomenon is expected for a saturable uptake process operating in the presence of substrates at supra-saturable concentrations.

Reduction of NMDAR currents is a result from the EAAT3-caused decrease in local glutamate concentrations

The above flow-stop results suggest that EAAT3 can regulate NMDAR current/activation. This EAAT3 function may be through a decrease in local glutamate concentrations by this transporter. To test this hypothesis, we superfused oocytes with a series of concentrations of glutamate and calculated the local glutamate concentrations sensed by the NMDAR based on the reversed Hill equation:

$$[\text{Glu}]_s = \frac{EC_{50}}{n \sqrt{\frac{I_{\text{max}}}{I} - 1}}, \quad (1)$$

where $[\text{Glu}]_s$ is the sensed glutamate concentrations, I is the glutamate-evoked current, I_{max} is the current response to a saturating glutamate concentration ($300 \mu\text{mol l}^{-1}$), and the EC_{50} and the Hill coefficient (n) refer to the mean values measured in oocytes expressing NMDAR only. As shown in Fig. 5, the $[\text{Glu}]_s$ was about one half to one third of the

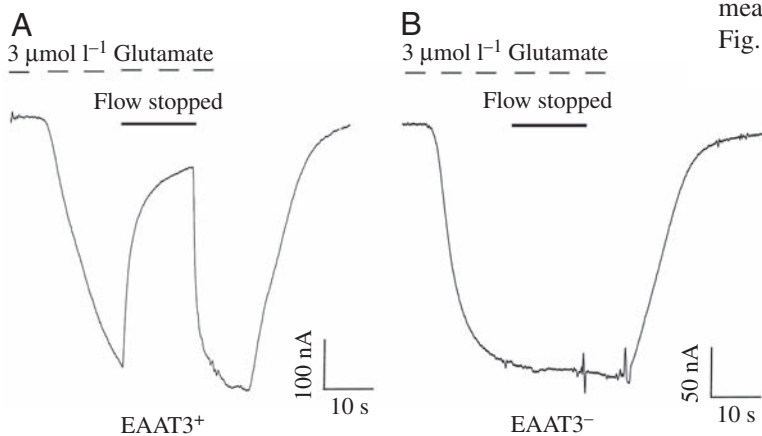


Fig. 2. Stopped-flow reduction of NMDAR current in EAAT3⁺ oocytes. NMDAR was activated continuously (dashed line) by a solution containing $3 \mu\text{mol l}^{-1}$ glutamate. Superfusate flow was stopped (solid line) for about 15 s during the activation. (A) Glutamate-induced current was decreased when the flow was stopped in EAAT3⁺ oocytes, (B) whereas the interruption of the flow had no effect on glutamate-induced current in EAAT3⁻ oocytes. Restarting the flow for 5 s restored the full amplitude of the glutamate-induced current in EAAT3⁺ oocytes.

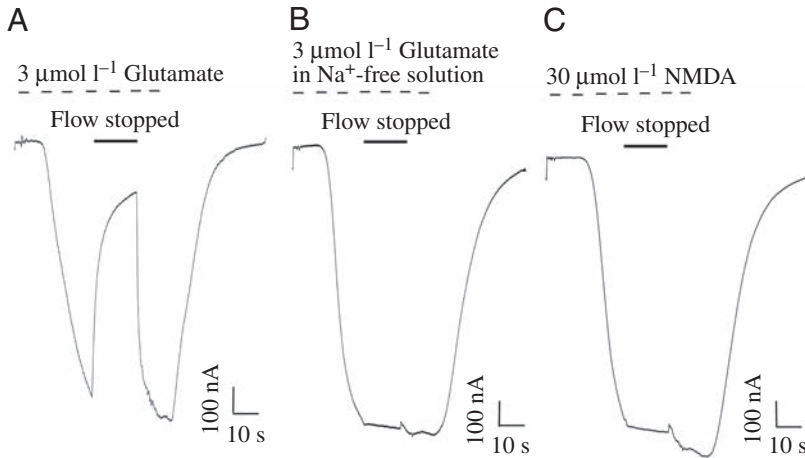


Fig. 3. Stopped-flow reduction of NMDAR current in EAAT3⁺ oocytes requires function of EAAT3. Stopped-flow reduction of NMDAR current (A) was not observed in EAAT3⁺ oocytes when Na⁺ was replaced by Li⁺ in the superfusates (B) or when NMDA was the agonist for NMDAR in the superfusate (C).

glutamate concentrations (ranging from 0.3 to 30 $\mu\text{mol l}^{-1}$) in the superfusates. The [Glu]_s values were 0.10 ± 0.07 , 0.35 ± 0.18 , 1.41 ± 0.71 , 4.53 ± 2.52 , 13.85 ± 6.13 $\mu\text{mol l}^{-1}$ (means \pm S.D., $N=12$) for glutamate concentrations in the superfusates = 0.3, 1, 3, 10 and 30 $\mu\text{mol l}^{-1}$, respectively.

Reduction of NMDAR current in EAAT3⁺ oocytes under continuous flow condition

Under continuous flow condition, L-glutamate at concentrations lower than 30 $\mu\text{mol l}^{-1}$ (no saturating concentrations for NMDAR) induced smaller currents in co-expressing oocytes than those in oocytes expressing NMDAR only (Fig. 6). This reduction can be quantitatively expressed as lower local glutamate concentrations being sensed by NMDAR in co-expressing oocytes than those in oocytes expressing NMDAR only (Fig. 5).

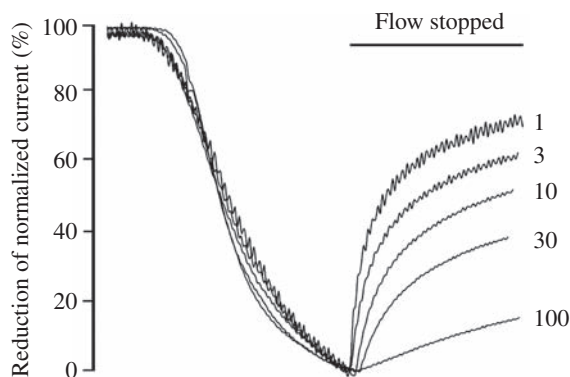


Fig. 4. Dose-dependence of the stopped-flow reduction of NMDAR current in EAAT3⁺ oocytes. Various concentrations (numbers on the right-hand side of the graph, $\mu\text{mol l}^{-1}$) of glutamate were applied to the oocytes and the current traces were normalized to the trace induced by 100 $\mu\text{mol l}^{-1}$ glutamate.

This reduction can also be quantitatively expressed as EC₅₀ being right-shifted (Fig. 7). As a control study, no EC₅₀ shift of the EAAT3 responses was noticed between the oocytes expressing EAAT3 only (31.8 ± 6.5 $\mu\text{mol l}^{-1}$, $N=12$) and co-expressing oocytes (30.9 ± 6.0 $\mu\text{mol l}^{-1}$, $N=9$, $P>0.05$) (Fig. 7A). In addition, no EC₅₀ shift was observed between the oocytes expressing NMDAR only (32.0 ± 9.8 $\mu\text{mol l}^{-1}$, $N=6$) and co-expressing oocytes (30.4 ± 5.2 $\mu\text{mol l}^{-1}$, $N=6$, $P>0.05$) when NMDA was used as the agonist for NMDAR (Fig. 7B). However, significant EC₅₀ shift was found between the oocytes expressing NMDAR only (oocytes injected with 40 ng mRNA of NMDAR, 3.6 ± 0.9 $\mu\text{mol l}^{-1}$, $N=16$) and co-expressing oocytes (oocytes injected with 40 ng mRNA of NMDAR and 40 ng mRNA of EAAT3, 10.5 ± 4.3 $\mu\text{mol l}^{-1}$, $N=15$, $P<0.05$) when glutamate was used as the agonist for NMDAR (Fig. 7C). The EC₅₀ was shifted even more in

oocytes that had higher quantitative ratio of EAAT3 proteins/NMDAR proteins (oocytes injected with 10 ng mRNA of NMDAR and 40 ng mRNA of EAAT3, 17.1 ± 6.2 $\mu\text{mol l}^{-1}$, $N=10$, $P<0.05$ compared with oocytes injected with 40 ng mRNA of NMDAR and 40 ng mRNA of EAAT3) (Fig. 7C). A linear correlation between the EC₅₀ of glutamate-induced current responses and the ratio of EAAT3 current/total glutamate-induced current in the co-expression oocytes was apparent (Fig. 7D). However, the EC₅₀ shift was abolished when Na⁺ in the superfusates was replaced by Li⁺ in the co-expressing oocytes injected with 40 ng mRNA of NMDAR and 40 ng mRNA of EAAT3 (3.8 ± 1.2 $\mu\text{mol l}^{-1}$, $N=6$,

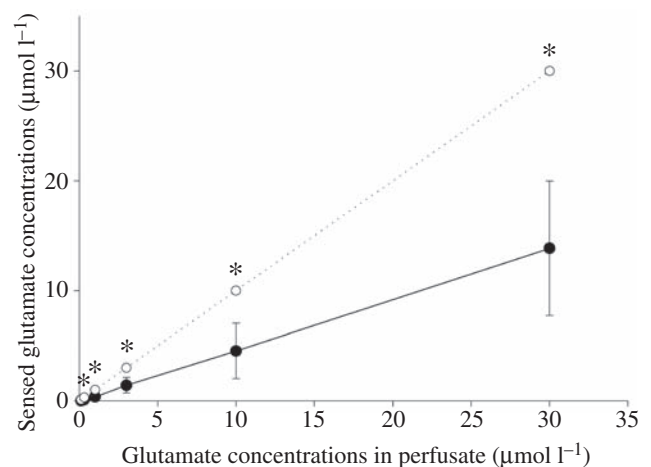


Fig. 5. Comparison of glutamate concentrations sensed by NMDAR (solid line) with those in the superfusates in the co-expressing oocytes. The dashed line refers to the situation that when the glutamate concentrations sensed by NMDAR equal to the glutamate concentrations in the superfusates. The sensed glutamate concentrations are calculated based on equation (1). Results are means \pm S.D. of sensed glutamate concentrations ($N=12$).

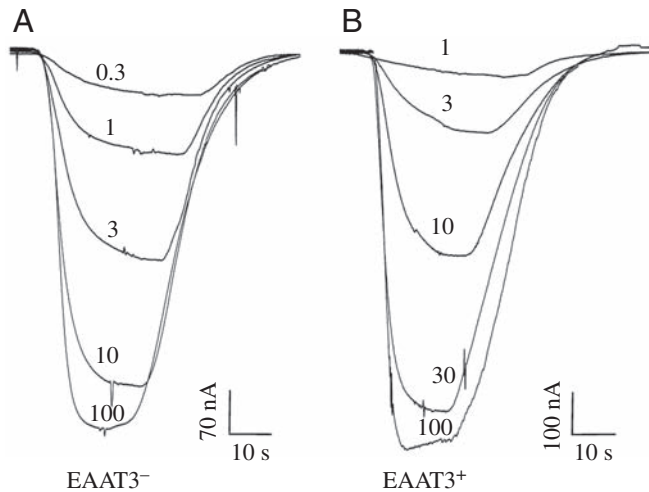


Fig. 6. Current traces showing that the reduction of glutamate-induced current under continuous flow conditions is established rapidly. The glutamate-induced current was produced repeatedly by 25 s applications of solutions containing various concentrations of glutamate (numbers in the figure, $\mu\text{mol l}^{-1}$) to oocytes expressing NMDAR only (EAAT3⁻ oocytes in panel A) or co-expression oocytes (EAAT3⁺ oocytes in panel B).

$P > 0.05$ compared with oocytes expressing NMDAR only) (Fig. 7C).

These results suggest that EAAT3 can decrease glutamate concentrations sensed by NMDAR in the co-expressing oocytes even under the continuous flow conditions, generating a steady-state glutamate concentration gradient between the bath solution and the cell membrane. The apparent affinities for glutamate in EAAT3⁺ oocytes appeared different from those in EAAT3⁻ oocytes even at early response time (Fig. 6), suggesting that the glutamate gradient was established as fast as the change of glutamate concentrations in solution.

Discussion

Control of NMDAR activation by EAAT3

Since both NMDAR and EAAT3 are expressed in the post-synaptic membrane *in vivo*, we simulated this situation by co-expressing these two proteins in *Xenopus* oocytes. In mature *Xenopus* oocytes, it was estimated that the membrane area is about eight times larger than that expected for a smooth sphere of the same apparent diameter (Supplisson and Bergman, 1997). Morphologically, this phenomenon is due to the presence of numerous small microvilli ($6\text{--}7 \mu\text{m}^2$) at the oolemma (Zampighi et al., 1995). These features of oocytes provide us a useful model since in many biological systems including synapses, the solute molecules *en route* to the membrane need to overcome diffusion barriers that produce small compartments where fine regulation of the concentrations of the molecules can take place. Near oocyte plasma membrane, the uptake of a molecule is counterbalanced by the passive diffusion of the molecule in the bath solution to form a concentration gradient across a diffusion barrier. In

oocytes, the vitelline envelope is $1\text{--}5 \mu\text{m}$ thick and produces an apparent unstirred layer of $d \approx 11 \mu\text{m}$, which limits rapid exchange of molecules between bath solution and the space near oocyte membrane (Costa et al., 1994; Supplisson and Bergman, 1997).

Our results showed that EAAT3⁺ oocytes had a fast recovery of NMDAR current in the stopped-flow experiments and a smaller NMDAR current in response to a non-saturating concentration of glutamate in the continuous flow experiments than EAAT3⁻ oocytes. These results suggest that EAAT3 regulates NMDAR currents. Previous studies have demonstrated that the application of EAAT inhibitors in brain slices or cell cultures increased the peak glutamate concentration in the synaptic cleft and prolonged EPSC decay at some synapses (Barbour et al., 1994; Diamond and Jahr, 1997; Kinney et al., 1997; Mennerick and Zorumski, 1994; Otis et al., 1996; Takahashi et al., 1995; Tong and Jahr, 1994). The action of EAATs at controlling the amplitude of EPSC has also been reported (Diamond, 2001; Diamond and Jahr, 1997; Tong and Jahr, 1994). Thus, EAATs, by regulating glutamate concentration in synapses, modulate glutamate neurotransmission such as that through NMDAR.

However, the modulation of glutamate neurotransmission by EAATs is not effective in all synapses. For example, studies of small simple synapses like hippocampal Schaffer collateral to pyramidal cell synapses have showed that inhibition of EAATs did not change the decay of EPSC (Hestrin et al., 1990; Isaacson and Nicoll, 1993; Sarantis et al., 1993). This failure to modulate may be due to very few EAATs that are expressed nearby to the glutamate receptors or a small quantitative ratio of EAATs/glutamate receptors. Under these conditions, small amount of glutamate from the total glutamate pool will bind to EAATs and the inhibition of EAATs may not significantly change the amount of glutamate available to glutamate receptors. To experimentally model this situation, we compared the effects of EAATs on NMDAR current in oocytes with different expression ratios of EAAT3/NMDAR. Our results showed that oocytes with smaller ratio of EAAT3/NMDAR had smaller changes in amplitude of NMDAR currents compared to oocytes with NMDAR alone. Consistent with our results, the synapses in which the inhibition of EAATs did not affect the EPSC usually have limited glial covering or large distance between the synaptic cleft and glial membrane (Lehre and Danbolt, 1998).

How do EAATs alter the availability of glutamate to glutamate receptors in synapses and, thus, regulate glutamate neurotransmission? Each transport cycle of glutamate by EAATs consists of at least three stages: glutamate binding, glutamate translocation and an anion-conducting state (Billups et al., 1998; Grewer et al., 2000). It was calculated that the time constant for a complete cycle of transport at -80 mV and 22°C is approximately 70 ms, which is significantly slower than the estimated glutamate-decay time constant in hippocampal synapses ($\sim 1\text{--}2 \text{ ms}$) (Clements et al., 1992). This difference was predicted to be true also at the physiological temperature (Wadiche et al., 1995). Thus, whether EAATs really constitute

a major mechanism for removing released glutamate was questioned (Wadiche et al., 1995). However, binding and translocation of glutamate may have rapid kinetics (Mennerick et al., 1999; Tong and Jahr, 1994). It was estimated by applying the laser-pulse photolysis technique of caged glutamate with a time resolution of 100 μ s that glutamate translocation occurs

within a few milliseconds after being bound to EAAT3 (Greuer et al., 2000). Jahr and colleagues have proposed that the action of EAATs at controlling EPSC is a consequence of rapid buffering of glutamate by a high density of binding sites provided by EAATs near to glutamate receptors (Diamond and Jahr, 1997; Tong and Jahr, 1994).

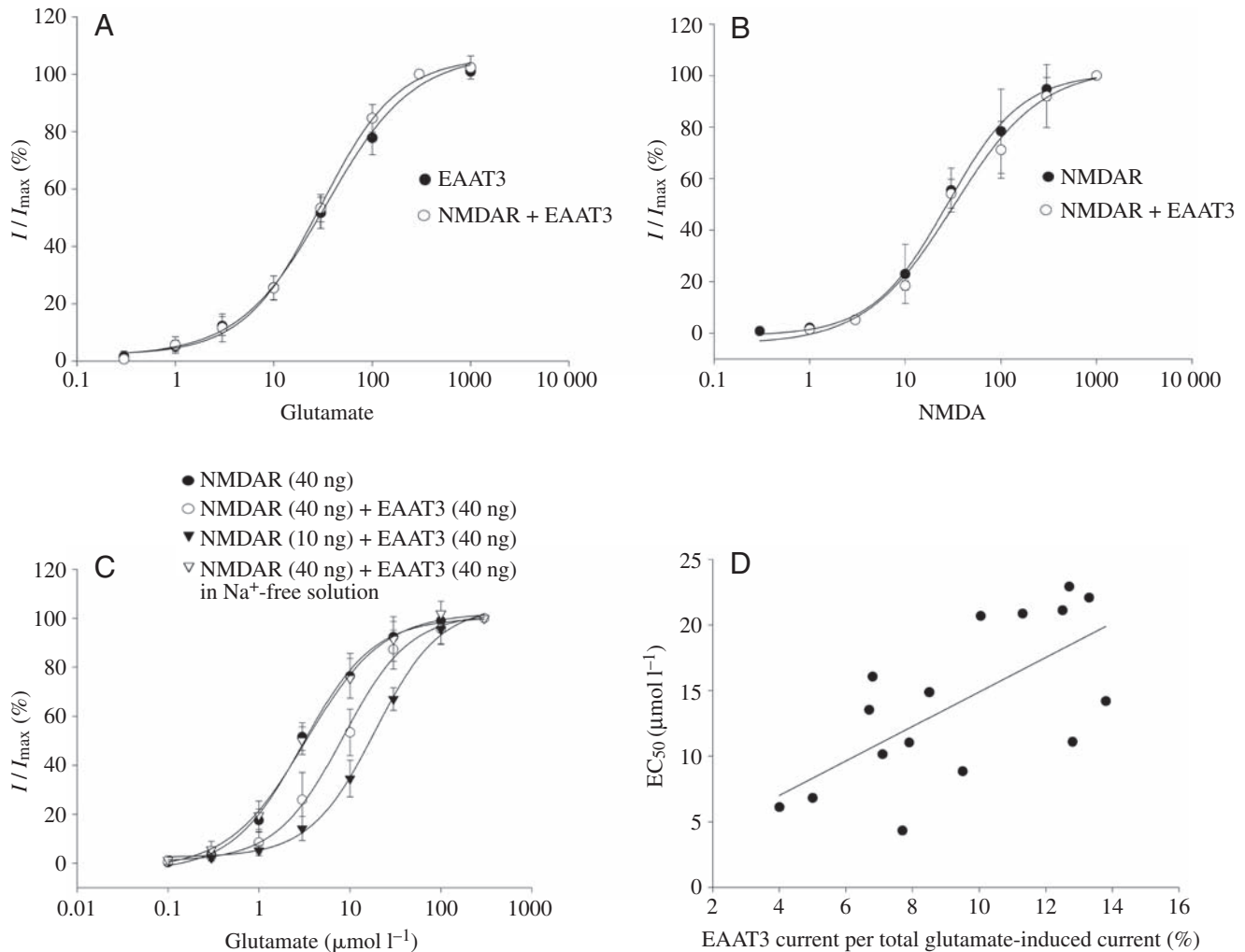


Fig. 7. Reduction of NMDAR current in EAAT3⁺ oocytes under continuous flow conditions. (A) Glutamate ($\mu\text{mol l}^{-1}$) dose–response curves of EAAT3. Oocytes were injected with 40 ng of EAAT3 mRNA (filled circle) or 40 ng of NMDAR mRNA and 40 ng of EAAT3 mRNA (co-expression, open circle). In co-expressing oocytes, glutamate was in a glycine-free solution containing 5 mmol l^{-1} Mg^{2+} to inhibit the NMDAR activation. Results are means \pm s.d. ($N=12$ for EAAT3 single expressing oocytes and $N=9$ for co-expressing oocytes) of the percentage of the currents induced by various concentrations of glutamate in the maximal EAAT3 current induced by 300 $\mu\text{mol l}^{-1}$ of glutamate (I_{max}). (B) NMDA ($\mu\text{mol l}^{-1}$) dose–response curves of NMDAR. Oocytes were injected with 40 ng of NMDAR mRNA (filled circle) or 40 ng of NMDAR mRNA and 40 ng of EAAT3 mRNA (open circle). In co-expressing oocytes, no EAAT inhibitors were included in the superfusates. Results are means \pm s.d. ($N=6$ for both NMDAR single expressing oocytes and co-expressing oocytes) of the percentage of the currents induced by various concentrations of glutamate in the maximal NMDAR current induced by 1 mmol of NMDA (I_{max}). (C) Dose–response curves of glutamate-induced current. Oocytes were injected with 40 ng of NMDAR mRNA (filled circle) or 40 ng of NMDAR mRNA and 40 ng of EAAT3 mRNA (open circle and open triangle) or 10 ng of NMDAR mRNA and 40 ng of EAAT3 mRNA (filled triangle). EAAT3 activity was inhibited by replacing Na^+ with Li^+ in the solution and the data are presented as open triangle in the graph. Results are means \pm s.d. ($N=16$ for filled circle, $N=15$ for open circle, $N=10$ for filled triangle and $N=6$ for the open triangle) of the percentage of the currents induced by various concentrations of glutamate in the maximal current induced by 300 $\mu\text{mol l}^{-1}$ of glutamate (I_{max}). (D) Relationship between the EC_{50} shift of the dose–response curves of glutamate-induced current and the percentage of EAAT3 current in the total glutamate-induced current in the co-expressing oocytes. EAAT3 current was isolated from NMDAR current by using a glycine-free solution containing 5 mmol l^{-1} Mg^{2+} to inhibit the NMDAR activation/current. Each data point represents data from one oocyte. A linear regression yielded an $r=0.607$ ($P<0.05$).

Implications

As discussed in the above section, studies in the literature have suggested the regulation of the amplitude of EPSC by EAATs. However, those studies were performed with use of various combinations of inhibitors to isolate glutamate receptor-mediated EPSC from that caused by other neurotransmitters. In addition, due to the lack of non-transportable inhibitors, most of those early studies were performed with transportable EAAT inhibitors. These EAAT inhibitors can induce glutamate release from intracellular space *via* heteroexchange (Volterra et al., 1996). Thus, the use of these inhibitors may have resulted in overestimation of the EAAT roles in maintaining extracellular glutamate homeostasis. To overcome this problem, Jabaudon et al. (1999) applied DL-threo- β -benzyloxyaspartate (TBOA), a non-transportable EAAT inhibitor developed recently, to rat hippocampal slice culture and used the NMDAR of patched CA3 hippocampal neurons as 'glutamate sensors'. They found that under basal conditions, the activity of EAATs compensates for the continuous, non-vesicular release of glutamate from the intracellular compartment. The inhibition of EAAT activity by TBOA immediately results in significant accumulation of extracellular glutamate (Jabaudon et al., 1999). The inhibition of postsynaptic EAATs in CA1 pyramidal cells by TBOA has been shown to enhance the activation of NMDAR by neurotransmitter spillover from neighboring synapses onto the synapses of these pyramidal cells (Diamond, 2001). We co-expressed NMDAR and EAAT3 in oocytes and did not need to use inhibitors to isolate NMDAR responses for study.

Our results showed that the effects of EAAT3 on the NMDAR activation decreased as the extracellular glutamate concentration increased. The effects were minimal at 100 $\mu\text{mol l}^{-1}$ or higher concentrations of glutamate. Although glutamate concentration in the synaptic cleft during excitation (which may vary in different synapses) is not known, it is believed that the concentration is in micro molar level (Danbolt, 2001). Thus, the *in vivo* glutamate concentrations in the synaptic cleft may fall into the concentration range that can be regulated by EAATs to effectively modulate glutamate receptor activation as demonstrated in our study. However, the degree of the effects of EAATs on glutamate receptor activation *in vivo* is obviously dependent on the density of EAATs and the distance between EAATs and glutamate receptors. The density of heterologous expression of transporters in oocytes usually is at about 150–3000 transporters μm^2 (Mager et al., 1993; Supplisson and Bergman, 1997; Zampighi et al., 1995). This density is lower than that estimated in the nervous tissue because 15,000 and 21,000 glial EAAT molecules were calculated to be present per μm^2 in the stratum radiatum of hippocampus CA1 and molecular layer of cerebellum, respectively (Lehre and Danbolt, 1998), and EAAT4 is at an average density of \sim 2000 molecules μm^2 of the molecular layer (Dehnes et al., 1998). Thus, the effects of EAATs on glutamate receptor activation under *in vivo* conditions may be bigger than that in our study. However, the

distance between EAATs and glutamate receptors *in vivo* may be larger than that in oocytes (we assume that NMDAR and EAAT3 proteins are distributed evenly at the surface of the oocyte including the microvilli). Only can EAATs located inside the synaptic cleft regulate glutamate concentrations there. However, all five EAATs cloned so far appear to be present outside synaptic cleft except for EAAT4 on the postsynaptic densities of Purkinje cell spines (Danbolt, 2001). The perisynaptic distribution of neuronal EAATs such as EAAT3 may limit glutamate diffusing into synapses from outside (Diamond, 2001).

Abbreviations

AMPA	α -amino-3-hydroxy-5-methy-4-isoxazol propionic acid
EAAT	excitatory amino acid transporters
EPSC	excitatory post-synaptic current
NMDA	N-methy-D-aspartate
NMDAR	N-methy-D-aspartate receptor
PDC	L-trans-pyrrolidine-2,4-dicarboxylate
TBOA	DL-threo- β -benzyloxyaspartate.

This study was supported by a New Investigator Award from the Foundation for Anesthesia Education and Research/Baxter Healthcare Corporation (Z.Z.), a grant from the National Institutes of Health RO1 GM065211 (Z.Z.) and by the Department of Anesthesiology, University of Virginia. We thank Stephen F. Heinemann (The Salk Institute for Biological Studies, San Diego, CA, USA) for providing the rat NMDAR1-1a and NMDAR2A constructs, Mattias A. Hediger (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA, USA) for providing the rat EAAT3 cDNA construct.

References

- Arriza, J. L., Eliasof, S., Kavanaugh, M. P. and Amara, S. G. (1997). Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc. Natl. Acad. Sci. USA* **94**, 4155-4160.
- Barbour, B., Keller, B. U., Llano, I. and Marty, A. (1994). Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. *Neuron* **12**, 1331-1343.
- Billups, B., Rossi, D., Oshima, T., Warr, O., Takahashi, M., Sarantis, M., Szatkowski, M. and Attwell, D. (1998). Physiological and pathological operation of glutamate transporters. *Prog. Brain Res.* **116**, 45-57.
- Clements, J. D., Lester, R. A., Tong, G., Jahr, C. E. and Westbrook, G. L. (1992). The time course of glutamate in the synaptic cleft. *Science* **258**, 1498-1501.
- Costa, A. C. S., Patrick, J. and Dani, J. (1994). Improved technique for studying ion channels expressed in *Xenopus* oocytes, including fast superfusion. *Biophysical J.* **67**, 395-401.
- Danbolt, N. C. (2001). Glutamate uptake. *Progr. Neurobiol.* **65**, 1-105.
- Dehnes, Y., Chaudhry, F. A., Ullensvang, K., Lehre, K. P., Storm-Mathisen, J. and Danbolt, N. C. (1998). The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. *J. Neurosci.* **18**, 3606-3619.
- Diamond, J. S. (2001). Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J. Neurosci.* **21**, 8328-8338.
- Diamond, J. S. and Jahr, C. E. (1997). Transporters buffer synaptically

- released glutamate on a submillisecond time scale. *J. Neurosci.* **17**, 4672-4687.
- Do, S.-H., Fang, H. Y., Ham, B. M. and Zuo, Z.** (2002a). The effects of lidocaine on the activity of glutamate transporter EAAT3: the role of protein kinase C and phosphatidylinositol 3-kinase. *Anesthesia Analgesia* **95**, 1263-1268.
- Do, S.-H., Kamatchi, G. L. and Durieux, M. E.** (2001). The effects of isoflurane on native and chimeric muscarinic acetylcholine receptors: the role of protein kinase C. *Anesthesia Analgesia* **93**, 375-381.
- Do, S.-H., Kamatchi, G. L., Washington, J. M. and Zuo, Z.** (2002b). Effects of volatile anesthetics on glutamate transporter, excitatory amino acid transporter type 3. *Anesthesiology* **96**, 1492-1497.
- Durieux, M. E.** (1993). An IBM-compatible software system for electrophysiologic receptor studies in *Xenopus* oocytes. *Comp. Meth. Programs Biomed.* **41**, 101-105.
- Fang, H., Huang, Y. and Zuo, Z.** (2002). The different responses of rat glutamate transporter type 2 and its mutant (tyrosine 403 to histidine) activity to volatile anesthetics and activation of protein kinase C. *Brain Res.* **953**, 255-264.
- Grewer, C., Watzke, N., Wiessner, M. and Rauen, T.** (2000). Glutamate translocation of the neuronal glutamate transporter EAAC1 occurs within milliseconds. *Proc. Natl. Acad. Sci. USA* **97**, 9706-9711.
- Hestrin, S., Sah, P. and Nicoll, R. A.** (1990). Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron* **5**, 247-253.
- Isaacson, J. S. and Nicoll, R. A.** (1993). The uptake inhibitor L-trans-PDC enhances responses to glutamate but fails to alter the kinetics of excitatory synaptic currents in the hippocampus. *J. Neurophysiol.* **70**, 2187-2191.
- Jabaudon, D., Shimamoto, K., Yasuda-Kamatani, Y., Scanziani, M., Gahwiler, B. H. and Gerber, U.** (1999). Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. *Proc. Natl. Acad. Sci. USA* **96**, 8733-8738.
- Kinney, G. A., Overstreet, L. S. and Slater, N. T.** (1997). Prolonged physiological entrapment of glutamate in the synaptic cleft of cerebellar unipolar brush cells. *J. Neurophysiol.* **78**, 1320-1333.
- Lehre, K. P. and Danbolt, N. C.** (1998). The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. *J. Neurosci.* **18**, 8751-8757.
- Mager, S., Naeve, J., Quick, M., Labarca, C., Davidson, H. and Davidson, L.** (1993). Steady states, charge movements, and rates for a cloned GABA transporter expressed in *Xenopus* oocytes. *Neuron* **10**, 177-188.
- Mennerick, S., Shen, W., Xu, W., Benz, A., Tanaka, K., Shimamoto, K., Isenberg, K. E., Krause, J. E. and Zorumski, C. F.** (1999). Substrate turnover by transporters curtails synaptic glutamate transients. *J. Neurosci.* **19**, 9242-9251.
- Mennerick, S. and Zorumski, C. F.** (1994). Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature* **368**, 59-62.
- Otis, T. S., Wu, Y. C. and Trussell, L. O.** (1996). Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. *J. Neurosci.* **16**, 1634-1644.
- Roskoski, R., Jr** (1979). Net uptake of aspartate by high-affinity rat cortical synaptosomal transport system. *Brain Res.* **160**, 85-93.
- Rothstein, J. D., Dykes-Hoberg, M., Pardo, C. A., Bristol, L. A., Jin, L., Kuncl, R. W., Kanai, Y., Hediger, M. A., Wang, Y., Schielke, J. P. et al.** (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16**, 675-686.
- Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N. and Kuncl, R. W.** (1994). Localization of neuronal and glial glutamate transporters. *Neuron* **13**, 713-725.
- Sarantis, M., Ballerini, L., Miller, B., Silver, R. A., Edwards, M. and Atwell, D.** (1993). Glutamate uptake from the synaptic cleft does not shape the decay of the non-NMDA component of the synaptic current. *Neuron* **11**, 541-549.
- Supplisson, S. and Bergman, C.** (1997). Control of NMDA receptor activation by a glycine transporter co-expressed in *Xenopus* oocytes. *J. Neurosci.* **17**, 4580-4590.
- Takahashi, M., Kovalchuk, Y. and Attwell, D.** (1995). Presynaptic and postsynaptic determinates of EPSC waveform at cerebellar climbing fiber and parallel fiber to Purkinje cell synapses. *J. Neurosci.* **15**, 5693-5702.
- Tong, G. and Jahr, C. E.** (1994). Block of glutamate transporters potentiates postsynaptic excitation. *Neuron* **13**, 1195-1203.
- Volterra, A., Bezzi, P., Rizzini, B. L., Trotti, D., Ullensvang, K., Danbolt, N. C. and Racagni, G.** (1996). The competitive transport inhibitor L-trans-pyrrolidine-2, 4-dicarboxylate triggers excitotoxicity in rat cortical neuron-astrocyte co-cultures via glutamate release rather than uptake inhibition. *Eur. J. Neurosci.* **8**, 2019-2028.
- Wadiche, J. I., Arriza, J. L., Amara, S. G. and Kavanaugh, M. P.** (1995). Kinetics of a human glutamate transporter. *Neuron* **14**, 1019-1027.
- Zampighi, G. A., Kreman, M., Boorer, K. J., Loo, D. D., Bezanilla, F., Chandy, G., Hall, J. E. and Wright, E. M.** (1995). A method for determining the unitary functional capacity of cloned channels and transporters expressed in *Xenopus laevis* oocytes. *J. Membr. Biol.* **148**, 65-78.
- Zuo, Z.** (2001). Isoflurane enhances glutamate uptake via glutamate transporters in rat glial cells. *Neuroreport* **12**, 1077-1080.